

EXHIBIT 3

REFERENCES

1. Abramson E, Wilson D, Arky RA: Rhinocerebral phycomycosis in association with diabetic ketoacidosis: report of two cases and a review of clinical and experimental experience with amphotericin B therapy. *Ann Intern Med* 66:735-742, 1967
2. Battock DJ, Grausz H, Bobrowsky M, et al: Alternate-day amphotericin B therapy in the treatment of rhinocerebral phycomycosis (mucormycosis). *Ann Intern Med* 68:122-137, 1968
3. La Touche CJ, Sutherland TW, Telling M: Rhinocerebral mucormycosis. *Lancet* 2:811-813, 1963
4. Gass JDM: Acute orbital mucormycosis: report of two cases. *Ophthalmol* 65:214-220, 1961
5. Baker RD: Mucormycosis — a new disease? *JAMA* 163:1014, 1957
6. Georgiade N, McGuire C, Crawford H, et al: Mucormycosis: palatal sloughs in diabetics. *Plast Reconstr Surg* 17:473, 1956
7. Bauer H, Flanagan JF, Sheldon WH: The effects of metabolic alterations on experimental rhizopus oryzae (mucormycosis) infection. *Yale J Biol Med* 29:23-32, 1956

SEMINARS IN MEDICINE
OF THE
BETH ISRAEL HOSPITAL, BOSTON



LOUIS M. SHERWOOD, M.D., *Editor*
EDITH E. PARRIS, *Assistant Editor*

TUMOR ANGIOGENESIS: THERAPEUTIC
IMPLICATIONS

JUDAH FOLKMAN, M.D.

THE growth of solid neoplasms is always accompanied by neovascularization. This new capillary growth is even more vigorous and continuous than a similar outgrowth of capillary sprouts observed in fresh wounds or in inflammation.¹ Many workers have described the association between growing solid malignant tumors and new vessel growth.²⁻⁴ However, it has not been appreciated until the past few years that the population of tumor cells and the population of capillary endothelial cells within a neoplasm may constitute a highly integrated ecosystem. In this ecosystem the mitotic index of the two cell populations may depend upon each other. Tumor cells appear to stimulate endothelial-cell proliferation, and endothelial cells may have an indirect effect over the rate of tumor growth.

TUMOR CELLS STIMULATING ENDOTHELIAL CELLS

Unlike a skin graft, which sends out capillary sprouts to join those that are advancing from the recipient, solid tumor implants must elicit all new vessels from the host. Time-lapse movies of the hamster cheek pouch show the rapid march of capillary sprouts toward a newly implanted tumor nod-

ule.⁵ Within three days new capillaries penetrate the tumor implant, and blood flow is established.

The rapidity with which tumor implants are able to stimulate cell division in neighboring capillary endothelial cells is illustrated in the experiments of Wood.⁶ Tumor cells injected into the artery supplying the ear chamber of a rabbit were observed as they entered the capillaries, traversed the capillary wall and arrived in the extravascular space, where the cells formed a microscopic tumor nodule. However, only 18 hours after their arrival, endothelial-cell regeneration and the formation of new capillary sprouts were observed to originate in neighboring postcapillary venules. Our own experiments have demonstrated the induction of DNA synthesis in previously resting endothelial cells of capillaries and venules within 2 to 3 mm of a microimplant of less than 1 million cells.⁷ This burst of regeneration of endothelium was observed as early as six hours after the injection of tumor cells. Electron microscopical autoradiographic sections showed incorporation of ³H-thymidine in endothelial cells of venules and capillaries surrounding the tumor implant. No tumor cells were present in any field, nor were there inflammatory cells. Many sections were taken from areas at least 3 mm from any tumor cell. This rapid stimulation of endothelial regeneration is not observed with dead tumor cells, or in control experiments in which saline or inflammatory agents (such as 0.2 per cent formic acid) are injected.

New capillary sprouts are elicited, even if a tumor implant is enclosed in a Millipore filter chamber. Greenblatt and Shubik⁸ have shown that vasoproliferative activity is consistently seen in the hamster cheek pouch adjacent to tumor implants despite separation of the tumor and its stroma by a Millipore filter that prevents the passage of cells. Ehrmann⁹ saw the same phenomenon with chorioncarcinoma in the hamster cheek pouch. We have demonstrated a similar effect, using Millipore chambers implanted on the fascial expanse of the dorsal air sac of the rat.

Taken together, these studies suggest that some diffusible message is released from tumor to nearby endothelial cells; these cells are then switched from a previously resting, nonregenerating state to a rapidly dividing group of regenerating cells capable of forming new capillary sprouts that can grow at the rate of 1 mm per day.

From the Department of Surgery, Children's Hospital Medical Center and Harvard Medical School, Boston, Massachusetts 02115.

Supported by a grant (5 R01 CA08185-06) from the National Cancer Institute, a grant from the American Cancer Society, National Chapter (IC-28), and gifts from the Merck Company and the Alza Corporation.

CAPILLARY ENDOTHELIAL CELLS MODULATING TUMOR CELLS

The other side of the equation reveals that solid tumors are far more dependent upon new capillary growth than we had previously believed. In the absence of neovascularization, most solid tumors stop growing when they are 2 to 3 mm in size and enter a dormant though viable state. This phenomenon can be observed *in vitro* with isolated perfused organs. Tumors implanted into these organs grow capillary in a diameter of 2 to 3 mm and then stop growing since they are unable to be vascularized because of artifacts unique to perfusion.^{12,13} A similar situation *in vivo* is observed when tiny tumors are implanted for more than one year in the anterior chamber of the eye of a guinea pig; these tumors do not enlarge beyond 2 to 3 mm.¹⁴ The tumors that exist in this dormant state have not become vascularized. When the tumors are removed from the eye after one year of this dormant existence and are then implanted in the muscle of a rabbit, however, rapid neovascularization is accompanied by rapid growth.

Even after a tumor has become vascularized the capillary endothelium that penetrated it has an indirect influence over the rate of tumor growth. Tannock^{15,16} has shown that the probability of a tumor cell entering mitosis decreases with increasing distance of the cell from its nearest capillary. Thus, even after vascularization has been established, the efficiency of diffusion of nutrients and wastes diminishes with increasing distance from each capillary. For example, in the mouse mammary tumor, the turnover time of the endothelial-cell population (50 hours) lags behind that of the tumor cells (22 hours). Consequently, as the tumor grows, the tardy proliferation of capillary endothelial cells leads to an increase in the intercapillary distance. Proliferation is diminished in tumor cells at the greatest distance from a capillary, which leads to a decreased rate of growth of the entire tumor. Therefore, Tannock^{15,16} has suggested that the rate of proliferation of endothelial cells may indirectly limit the rate of tumor growth.

From this information we can form a working hypothesis not only that endothelial cells may limit tumor expansion but also that without neovascularization, solid tumors might become completely dormant at a diameter of 2 to 3 mm. Solid tumors can grow to visibility only if they can vascularize themselves. Therefore, the mechanism by which tumor implants stimulate neovascularization must be well understood before therapy based upon interference with angiogenesis can be devised.

TUMOR-ANGIOGENESIS FACTOR (TAF)

We have isolated a factor from human and animal tumors (tumor-angiogenesis factor or [TAF]) that is mitogenic in endothelial cells and stimulates rapid

formation of new capillaries in animals.¹⁷ TAF is not found in normal tissues with the exception of placenta.

To isolate TAF, tumor cells are harvested from the ascites or solid phase, separated from red cells, washed in buffer and disrupted by exposure to nitrogen (900 lb per square inch) for 30 minutes, after which the pressure is suddenly released. The nuclei are separated, and the remaining cellular components are sedimented by centrifugation at 260,000 \times g. This sediment is delipidated, trypsinized and then purified by gel filtration on Sephadex-G 100. Fraction II of the eluate contains angiogenesis activity (Fig. 1). The biologic activity is assayed by intermittent injection of the fraction over a period of 48 hours into the dorsal air sac of a rat through a tiny cylindrical Millipore filter. Although reliable, the assay system is cumbersome and consumes large quantities of TAF. The few micrograms of TAF obtained from 1×10^6 tumor cells are necessary to produce a detectable reaction in one rat. We are now developing short-term cultures of endothelium that may respond to smaller concentrations of TAF by increased incorporation of ³H-thymidine. The isolation procedure must also be carried out under sterile conditions, because the slightest bacterial growth during the assay can generate products that cause background inflammation.

Tissues in the air sac exposed to inactivated TAF or other nonactive biologic fluids (control fluids) show normal vascularity. After 24 hours of exposure to active TAF, however, the tissue beneath the filter contains clusters of new capillaries. After 48 hours of exposure the neovascularization is more intense, and the histologic sections show heavy capillary regeneration. The neovascularization is not due simply to vasodilatation or inflammation, since new-vessel growth is present. Histologic sections show frequent mitotic figures in regenerating endothelial

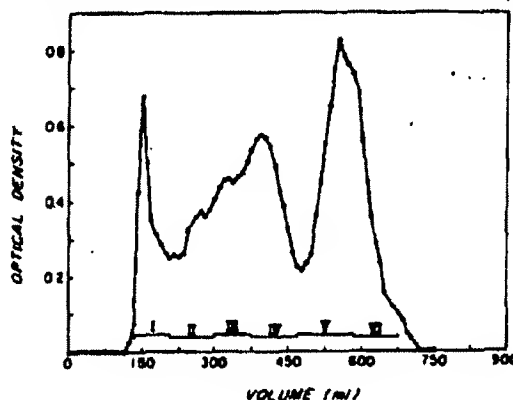


Figure 1. Separation on a G-100 Sephadex Column of the 360,000-G Pellet after Delipidation and Trypsin Digestion (Reproduced from Folkman et Al.¹⁷ with the Permission of the Publisher) — Elution with Sodium Chloride, 0.15 M. Optical Density at 280 nm vs. Eluting Volume.

cells that also incorporate ^3H -thymidine. There is almost no inflammatory infiltrate such as one sees in granulation tissue, and the reaction cannot be suppressed by steroids. By comparison, inflammation produced in the air sac by injection of formic acid (0.2 per cent) through the Millipore filter shows the usual inflammatory cellular exudate on histologic sections. Scattered neovascularization appears late, wherever capillaries are actually injured or necrosed by the formic acid. However, this neovascularization and the inflammation preceding it can be entirely eliminated with steroids.

TAF has not been completely characterized, but its molecular weight is approximately 100,000. It is rapidly destroyed by ribonuclease and by heating to 56°C , but it is unharmed by trypsin. TAF contains 25 per cent ribonucleic acid associated with about 10 per cent protein and 50 per cent carbohydrate. It also has a lipid component that apparently reduces angiogenesis activity. When the lipid coat is removed, TAF activity is enhanced, implying that the lipid may act as an outer coat for the TAF molecule.

TAF does not produce a permanent change in capillary endothelial cells, since withdrawal of TAF is followed by disappearance of newly formed capillaries. TAF has also been found in hepatoblastoma, Wilms's tumor, neuroblastoma and rhabdomyosarcoma (in nine children) as well as in Walker-256 carcinoma (both solid and ascites), mouse melanoma (B16) and choriocarcinoma (kindly supplied by Dr. Robert Ehrmann, of the Deaconess Hospital, Boston, Massachusetts). Therefore, TAF appears not to be species specific, and its primary target is the endothelial cell. Human tumor TAF causes mitogenesis in endothelial cells of the rat, mouse and rabbit.

If TAF is the mediator of tumor neovascularization, we can then hypothesize that most solid tumors, whether they originate from a single cell transformed by virus or carcinogen or whether they begin as a metastatic implant, must exist initially as a small population of cells dependent upon nutrients that diffuse from the extravascular space (Fig. 2). The pinpoint colony eventually expands to a size where simple diffusion of nutrients (and wastes) is insufficient. New capillaries are elicited, and the tumor then enters a phase in which perfusion becomes the mechanism by which nutrients arrive and metabolic wastes are carried away. It is possible that TAF is responsible for this final stage.

In terms of therapy, the most vulnerable period in the life of a solid tumor may be the brief interval before vascularization. If TAF could be blocked at this point, tumor growth might be arrested at a tiny diameter of 2 to 3 mm, since this appears to be the approximate diameter beyond which a packed population of tumor cells cannot expand without new capillaries. If this theory is correct, "anti-angio-

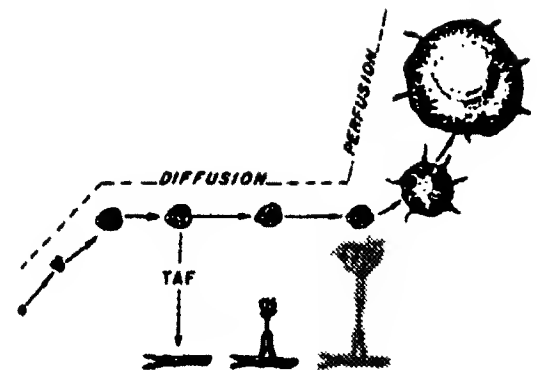


Figure 2. Illustration of the Concept That Most Solid Tumors May Exist Early as Tiny Cell Populations Living by Simple Diffusion in the Extracellular Space (Further Growth Requires Vascularization, and the Tumor Then Maintains Itself by Perfusion).

Tumor-angiogenesis factor (TAF) may be the mediator of neovascularization.

genesis" therapy, perhaps by immunization against TAF, should provide a powerful adjunct to the control of solid neoplasms.¹⁰

"ANTI-ANGIOGENESIS"

We have proposed the term "anti-angiogenesis" to mean the prevention of new vessel sprouts from penetrating into an early tumor implant. The term should not apply to the vasoconstriction or infarction of vessels already connected to a tumor. If a tumor could be held indefinitely in the nonvascularized dormant state, there are a number of theoretical benefits. For example, it is possible that metastases will not arise from a nonvascularized tumor. Recent evidence^{12,13} suggests that metastases diminish when the primary tumor is relatively devascularized. It is also probable that tiny unvascularized tumors may be far more vulnerable to chemotherapy than larger tumor masses. However, it is still not known whether the dormant state is maintained by a high rate of cell loss or by a decline in mitotic rate. A tiny unvascularized tumor implant might also be more susceptible to cell-mediated immunologic attack than a vascularized, rapidly growing tumor. Circulating antibody may be unable to reach an unvascularized tumor in as high a concentration, because of the diffusion gradient that would exist over the distance between the nearest capillaries and the tumor implant. Immune lymphocytes, because of their migratory ability, might easily reach the tumor implant, exposing it to the full force of cell-mediated attack without the protection of blocking antibody. Therefore, "anti-angiogenesis" may synergize immunotherapy.

There are some other interesting aspects of the problem that have therapeutic implications. We

ave quantified the extent of endothelial-cell regeneration in terms of capillary density for a variety of human tumors, and a striking stratification appears.¹¹ One thinks of the vascularity as a function of the endothelial-cell dependency of the parenchymal tumor cells, there is a hierarchy in which brain tumors appear to be the most dependent upon endothelial-cell proliferation, carcinomas slightly less, sarcomas less, and chondrosarcomas the least. Certain chondrosarcomas reach enormous dimensions; yet capillaries are sparse, and endothelial cells in these capillaries are not active. If this stratification is real, it is possible that "anti-angiogenesis" could be extremely important in the therapy of brain tumors, and carcinoma, and less effective for sarcoma and ineffective in chondrosarcoma.

Furthermore, the concept of "anti-angiogenesis" raises the important question of how angiogenesis information is transferred from malignant cells to endothelial cells. Finally, if blockade of angiogenesis results in a tiny dormant tumor, what is the mechanism of the dormant state? Can it be explained entirely by accumulation of inhibitory metabolites in the micro-environment of the tumor nodule?

Although evidence for these proposals is still largely indirect and fragmentary, it seems appropriate to speculate that "anti-angiogenesis" may provide a form of cancer therapy worthy of serious exploration. One approach to the initiation of "anti-angiogenesis" would be the production of an antibody against TAF; such an attempt is under way in our laboratory. We are not unaware of the difficulties involved in the production of antibody against ribonucleic acid. Nevertheless, by whatever technique "anti-angiogenesis" can be achieved, it may become a powerful adjunct to present methods of cancer therapy. If "anti-angiogenesis" is not possible, or even if the concept is wrong, the careful exploration of its consequences may reveal something fundamental about the behavior of tumor cells growing in a packed population in vivo.

DISCUSSION

DR. MICHAEL BACH: What results have you had in trying to produce antibodies against TAF?

DR. FOLKMAN: Several pieces of evidence suggest that we cannot yet immunize against it. First of all, if TAF is injected into a rabbit ear chamber, in 48 hours the entire chamber begins to fill with new vessels. That can be done repeatedly, and the animal does not appear to be immunized. Secondly, if one immunizes mice with TAF itself, nothing happens; tumors will grow and stimulate neovascularization. Thirdly, in a system like the Walker carcinoma, which grows in the rat, the animal can be immunized against the carcinoma by an injection of dead cells. One of our students, Mr. Joseph Corkery, has done this study. He has repeatedly immunized

rats so that they can accept millions of tumor cells and never grow a tumor. He can then take fresh Walker cells, isolate the TAF fraction, inject it and get beautiful vascularization.

DR. JOHN W. ROWE: If you radiate normal endothelium, can you change the sensitivity of these cells to tumor angiogenesis?

DR. FOLKMAN: That is a very good experiment, but we have not done it. There is some clinical evidence that over-radiation of the endothelial-cell population will prevent or delay future vascularization. It is interesting that many years after a tumor bed has been heavily radiated, a chondrosarcoma may appear. This tumor hardly requires any vascularization.

DR. FRANK DAVIDOFF: We have seen an analogous situation — that is, the revascularization of diabetic retina. I wonder if you have any information on the hormonal dependence of this host-vessel response to tumors.

DR. FOLKMAN: We do not know what factors modify tumor angiogenesis, except that steroids will not suppress it. Neovascularization that occurs without malignant change usually appears during the repair process of tissue injury. For example, in an animal if mild inflammation is produced with heat, no new vessels appear. If the intensity of the heat is increased so that there is inflammation and minimal necrosis, neovascularization is observed several days later. If the necrosis is extensive because of intense heat, onset of neovascularization may be delayed for weeks. The same effect can be produced with low pH (less than 3.0) and high osmolarity (greater than 1000 mOsm). I cannot guess what might be responsible for the revascularization in diabetes.

DR. PETER LAMBERT: Is TAF found in plasma?

DR. FOLKMAN: We have been unable to find it in plasma. When TAF is added to plasma, the angiogenesis activity disappears. It is possible that our assay is not sensitive enough to detect small amounts of TAF, which might be present in plasma.

DR. CARL HIRSCH: It is difficult to imagine that a tumor would have a unique property that was not at some time present in the host. What relation do you think it might have to normal host vascularization (for example, during regeneration), and have you examined embryonic or fetal tissue?

DR. FOLKMAN: Human placenta does have angiogenesis activity and in about the same fraction as we find it in tumor. However, we have been unable to isolate any angiogenesis activity from regenerating rat liver. I would guess that in the embryo, capillary endothelial turnover is proceeding at a maximal rate, and that this turnover is greatly reduced in the adult animal. Whether the stimulus for this turnover is repressed in the adult animal, but reappears in neoplastic cells, is open to speculation.

DR. ALFRED GOLDBERG: Does the factor have any effect on isolated tumor cells?

DR. FOLKMAN: I do not know.

DR. GOLDBERG: Do you have any quantitative information on the time course of appearance of TAF or its dependency on the quantity of tumor?

DR. FOLKMAN: I can tell you that from 3×10^6 tumor cells, we obtain approximately 10 mg of crude cytoplasmic TAF of which 25 per cent is ribonucleic acid. This is before delipidation and trypsinization and before Sephadex-gel purification.

DR. HOWARD H. HIATT: Have you looked at cultured cells for the factor?

DR. FOLKMAN: We worked with the CEM line of lymphoblastic leukemia obtained from Dr. George Foley. We could not isolate TAF from it and have used it as a negative control. With Dr. Michael Oxman we looked at hamster embryo cells in culture. We could not demonstrate any angiogenesis activity in these cells. Then the cells were transformed with SV40 virus, and they were still negative for TAF. We then injected these transformed cells into hamsters and obtained large, well vascularized tumors. Cells from the tumors did produce TAF. Then, when these cells were returned to culture, the assays were weakly positive after the first passage, but negative after that. These experiments are still in progress.

DR. JONATHAN GLASS: When you isolate your fraction, do you do so from the whole tumor or do you try to isolate tumor cells?

DR. FOLKMAN: Solid tumor can be used, but it must be passed through a cytosieve to obtain individual cells, and much effort is spent in getting rid of the fibrous tissue, the red cells and so forth. Most of the time we use ascites tumor cells.

DR. EDGAR HENSHAW: I wonder if you would actually find the factor in spontaneous human or animal tumors? Most of the tumors grow well at the periphery, and they are somewhat anoxic in the middle.

DR. FOLKMAN: When we attempt to isolate TAF from human tumors, we are careful to discard the necrotic portions, which are usually in the center. To my mind, the necrotic center of a large tumor was at an earlier time well vascularized. However, the enormous pressures that build up within a large tumor could diminish blood flow to the center.

A PHYSICIAN: Have there been any attempts to try this factor to help in the treatment of normal tissue? Does the factor convert normal tissue to tumor?

DR. FOLKMAN: It does not convert normal tissue to tumor. If one stops applying the material, the newly generated vessels regress in three or four days. Theoretically, this material might have a beneficial effect on a granulating bed in preparation for a skin graft.

DR. DAVIDOFF: Have you any thoughts about the site of TAF production in the tumor cell? It might be made on the surface, and, if so, do you think you could find it by washing off all the membranes?

DR. FOLKMAN: We have tried to obtain TAF by repeated washing of intact tumor cells, but without success. Our best results come from breaking up the cells. Most of our work so far has been done with TAF obtained from the cytoplasm. The mitochondrial fraction and microsomal fractions have always been negative. More recently, Dr. Dorothy Tian, in our laboratory, has found TAF activity in nuclear chromatin, but we do not yet know which component of the chromatin contains the activity.

REFERENCES

1. Algire GH, Chalkley HW: Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wound, and to normal and neoplastic transplants. *J Natl Cancer Inst* 6: 85, 1945
2. Feigin I, Allen LB, Lipkin L, et al: The endothelial hyperplasia of the cerebral blood vessels with brain tumors, and its sarcomatous transformation. *Cancer* 11:264-277, 1958
3. Ide AG, Baker NH, Warren SL: Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. *Am J Roentgenol* 42:891-899, 1939
4. Goodall CM, Feldman R, Sanders AG, et al: Vascular patterns of four transplantable tumors in the hamster (*Mesocricetus auratus*). *Angiology* 16:622-625, 1965
5. Chalkley HW: Discussion of Algire GH, Legallais FY: Growth and vascularization of transplanted mouse melanomas. The Biology of Melanomas: Proceedings of the 1st Conference on the Biology of Normal and Atypical Pigment Cell Growth. New York 1946. Edited by RW Miner, New York. Academy of Science, 1948, pp 164-165
6. Warren BA: The ultrastructure of capillary sprouts induced in melanoma transplants in the golden hamster. *J R Microsc Soc* 86: 177-187, 1966
7. Warren BA, Shubik P: The growth of the blood supply to melanoma transplants in the hamster cheek pouch. *Lab Invest* 15:464-478, 1966
8. Wood SJ Jr: Pathogenesis of metastasis formation observed in vivo in the rabbit ear chamber. *Arch Pathol* 66:550-568, 1958
9. Cavallo T, Couran R, Folkman J, et al: Tumor angiogenesis: rapid induction of endothelial regeneration demonstrated by autoradiography and electron microscopy. *J Natl Cancer Inst* (in press)
10. Greenblatt M, Shubik P: Tumor angiogenesis: transfer diffusion studies in the hamster by the transparent chamber technique. *J Natl Cancer Inst* 41:111-124, 1968
11. Ehrmann RL, Knott M: Choriocarcinoma: transfer stimulation of vasoproliferation in the hamster cheek pouch - studied by light and electron microscopy. *J Natl Cancer Inst* 41:1329-1344, 1968
12. Folkman J: The Intestine as an organ culture. *Carcinoma of the Colon and Antecedent Epithelium*. Edited by WJ Burdette. Springfield, Illinois, Charles C Thomas, 1970, pp 113-127
13. Gimbrone MA Jr, Aster RH, Couran RS, et al: Preservation of vascular integrity in organs perfused *in vitro* with a platelet medium. *Nature (Lond)* 222:33-36, 1969
14. Greene HSN: Heterologous transplantation of mammary tumors. I. The transfer of rabbit tumors to alien species. *J Exp Med* 73:461-474, 1941
15. Tannock IF: The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumor. *Br J Cancer* 22:258-273, 1968
16. Idem: Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. *Cancer Res* 30:2470-2476, 1970
17. Folkman J, Merier E, Atherly C, et al: Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* 133:275-288, 1971
18. Folkman J: "Anti-angiogenesis": new concept for therapy of tumors. *Ann Surg* (in press)
19. Salisbury AJ, Burrage K, Hellmann K: Inhibition of metastasis by I.C.R.F. 159: selective deletion of a malignant characteristic. *Br Med J* 4:344-346, 1970
20. Breslow A: Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg* 172:902-908, 1970
21. Brem S, Couran R, Folkman J: Tumor angiogenesis: a quantitative method for histologic grading. *J Natl Cancer Inst* (in press)